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FINAL REPORT

EFFECT OF ELK VELVET ANTLER ON BLOOD CHOLESTEROL LEVELS AND BLOOD CLOTTING DISORDERS

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Final Report

Title: Effect of Elk Velvet Antler on Blood Cholesterol Levels and Blood Clotting Disorders.

Project: 19990328 (Res. 44BX)

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Abstract/Summary:

The objective of this study was to examine the effects of orally administered elk velvet antler (EVA) on plasma cholesterol profiles and blood clotting times in hypercholesterolemic rabbits. Diets containing test agents, EVA, bone meal (control), fluvastatin (lipid lowering agent) and warfarin (anticoagulant), were started in hypercholesterolemic rabbits but the study was terminated prematurely when several animals became ill. Rabbits which received 100 mg/kg of EVA and fluvastatin appeared to be the most resilient to the illness. Analysis of plasma lipids and atherosclerotic plaques however showed no significant difference between test groups. Results suggest that 100 mg/kg EVA and fluvastatin may have a protective effect which is independent of lipid lowering action.

Executive Summary:

Project Outline:

Elk velvet antler (EVA) has been used in tradional chinese medicine for over 2000 years as a preventative medicine. Antler is thought to improve general cardiovascular health by lowering blood cholesterol levels, normalizing blood pressure, inhibiting coagulation (thinning) of the blood as well as stimulating the synthesis and release of red blood cells into circulation. In the western medical community, it has been well established that the risk of coronary artery disease increases when the level of cholesterol in the blood increases. Angina (a sudden and severe pain in the chest and left arm), stroke, heart attack and heart failure are a few cardiovascular diseases which can result from elevations in blood cholesterol levels.

The objective of this study was to examine the dose dependent effects of oral administration of EVA on plasma lipid profiles, development of athereosclerosis and blood clotting time in diet-induced hypercholesterolemic rabbits.

The effects of 3 separate doses of EVA, 10, 30 and 100 mg/kg body weight/day, were compared to 7 groups of control animals. Group 1 rabbits received regular rabbit feed without added cholesterol. Group 2 rabbits received regular rabbit feed with the addition of 1% cholesterol. Group 6, 7 and 8 were considered a sham control and were feed 10, 30 and 100 mg/kg body weight/day bone meal instead of EVA. Group 9 rabbits ate a feed with fluvastatin, a pharmaceutical cholesterol lower agent, incorporated. Group 10 rabbits ate a feed with warfarin, a pharmaceutical anticoagulant agent, incorporated. Each experimental group was comprised of 7 rabbits.

To establish high plasma cholesterol levels all rabbits, except those in group 1, were fed regular rabbit feed containing 1% added cholesterol for four weeks. No test agents were added during this period. At the end of the four week induction period, blood samples were taken from each animal. From these samples a complete blood cell count and lipid analysis were conducted along with three blood clotting assays. The blood count included the measurement of white blood cells (WBC), red blood cells (RBC), hemoglobin, hematocrit and platelets. Lipid analysis included total plasma cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol and a ratio of total cholesterol to HDL cholesterol. The three clotting assays were prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT).

Blood count analyses of blood samples obtained after the four week induction period showed no significant difference between the groups for RBC, hematocrit, hemoglobin or platelets. However , Group 1 (regular feed control group) had significantly lower WBC counts when compared to the other groups which received the 1% cholesterol diet. The rabbits showed no signs of infection, were eating and maintaining weight and appeared healthy. Lipid analysis revealed the expected significant increase in total cholesterol with control levels of 1.7 ± 2.28 mmol/L compared to a combined average of all other groups of 41.69 ± 23.43 mmol/L. HDL cholesterol was also significantly elevated in the 1% cholesterol groups with levels of 4.43 ± 2.31 mmol/L compared to $0.42 \pm .29$ mmol/L for group 1 rabbits. Triglycerides were not significantly elevated. All three coagulation assays also showed no significant difference between groups.

Following the four week induction period each test group received feed which included the incorporation of a specific test agent. The test agent feed was prepared based on the average rabbit weight of 3 kg and average feed consumption of 125 grams per day. The following list indicates which test agent each group received with doses being listed as mg dose per kg body weight per day.

Group 1	Control with regular rabbit feed
Group 2	1% cholesterol diet control
Group 3	10 mg/kg/day EVA in 1% cholesterol diet control
Group 4	30 mg/kg/day EVA in 1% cholesterol diet control
Group 5	100 mg/kg/day EVA in 1% cholesterol diet control
Group 6	10 mg/kg/day bone meal in 1% cholesterol diet control
Group 7	30 mg/kg/day bone meal in 1% cholesterol diet control
Group 8	100 mg/kg/day bone meal in 1% cholesterol diet control
Group 9	1.2 mg/kg/day fluvastatin
Group 10	0.1 mg/kg/day warfarin

The test diets were to be given for 12 weeks at which time blood samples were to be retaken and aortas excised for atherscleroticplaque assessment.

One week after starting the test phase, animal care techinicians involved in the study noticed four rabbits had stopped eating. From their overall appearance the suspected cause was a gastric trichobezoars (hairballs) or constipation. As the animals were not eating and therefore not consuming the test agents, they were removed from the study. The rabbits were from Group 1, 9 and two from Group 10. One week later 18 more animals were observed to have stopped eating and on the next day showed signs of jaundice. In an attempt to determine the cause of the jaundice these animals were sent to Prairie Diagnostic Services at the Western College of Verterinary Medicine for necropsy analysis. Examinations concluded that these rabbits had a macrocytic, regenerative anemia, colestasis and hepatic fibrosis. The anemia and hepatic fibrosis was not the result of infection as no signs of infection were observed and screens for infectious agents were negative. Initial toxin screens for coupounds known to produce anemia and hepatic fibrosis, such as Cu^{2+} or Zn^{2+} were also negative. Although high cholesterol levels are known to compromise both red blood cells by increasing membrane fragility and liver tissue by causing hepatic lipidosis, it is thought that an unidentified toxin may have exacerbated the red blood cell destruction and liver toxicity. The feed has been sent for toxicological screening to the Toxicology Center at the University of Saskatchewan.

The study was terminated after 9 weeks on the test diets with only 31 rabbits remaining in the study. All but two of the test groups were down to 3 or fewer animals. The only groups to have 5 of 7 animals remaining were Group 9 (fluvastatin) and Group 5 (100 mg/kg/day EVA). Although not a significant, these results may suggest some level of protection was provided by these agents.

Final blood sample analysis revealed an expected decrease in RBC counts, hemoglobin and hematocrit due to the hemolytic anemia. Lipid values remained significantly elevated with none of the treatment groups showing a therapeutic reduction in cholesterol values. As a result of the severe hemolytic anemia and small number of animals remaining in the study, coagulation assays were not preformed on the final blood samples. Aortas were removed and stained to assess lipid deposition. Densitometer analysis of stained lipid deposits showed no significant difference between treatment groups.

Although no reductions in plasma cholesterol levels or aortic lipid deposition were observed, rabbits which received high dose EVA or fluvastatin appeared to be least affected by the hemolytic anemia and liver toxicity. These results may suggest a possible protective affect provided by these two agents. A study has been undertaken in the laboratory of Dr. S. Hemmings in the Department of Physiology, University of Saskatchewan to assess the hepatoprotective effects orally administered EVA.

Evidence of a toxic agent as of yet have not been determined by feed toxicological analysis.

Technical Report:

Introduction:

Atherosclerosis of the coronary and peripheral vasculature is the leading cause of death in Canada accounting for 37% of total deaths (Heart and Stroke Foundation of Canada, 1999). Central to the pathogenesis of atherosclerosis is the deposition of cholesterol in the smooth muscle of the arterial wall (Levine et al., 1995; Williams et al., 1995). Cholesterol, a fat-like substance, is essential in the production and maintainance of nerve cells and as the central component of many natural hormones. Cholesterol is produced in the human body mainly by hepatic tissue but is also readily obtained by eating animal products such as, meats, eggs, milk and cheese. Like other fat substances cholesterol is insoluble in aqueous environments, such as the plasma, and therefore must be transported within a specialized molecule comprised of phospholipids and proteins. In healthy subjects these specialized carriers or lipoproteins function to distribute and recycle cholesterol. Very Low Density (VLDL) and Low Density Lipoproteins (LDL) transport cholesterol from the liver to peripheral tissue including arterial walls. Elevations of these transporters is correlated with increased risk of coronary artery disease. High Density Lipoproteins (HDL) carries cholesterol away from tissues, including the arterial wall, back to the liver and thus appears to be beneficial in lowering risk factors of coronary artery disease (Boon et al., 1995).

Atherosclerosis arises as circulating moncytes migrate into the smooth muscle wall and take up oxidised LDL from the plasma. These lipid-laden foam cells eventually die and release their lipid contents. Smooth muscle cells then migrate into and proliferate within this plaque. As the process continues the plaque grows, encroaching into the lumen of the blood vessel, affecting blood flow and if extensive blood pressure (Boon et al., 1995). In addition to the direct adverse hemodynamic effects of atherosclerosis, these plaques are also covered by a thick cap of collagen rich fibrous tissue which stimulates coagulation. Extensive thrombus development and embolism of the thrombus lead to complete compromise of blood flow and ischemic damage of surrounding tissue. This ischemia can be manifested as angina, myocardial infarction, arrythmias, heart failure and stroke (Boon et al., 1995). Effective lipid and blood coagulation therapy are therefore essential in effectively treating coronary artery disease.

In Saskatchewan alone, five million dollars are currently being spent annually on the purchase of only one class of cholesterol lowering agents for clinical therapy. Four classes of drugs are currently available (Saskatchewan Formulary,). Although effective these drugs do have adverse effects such as potentially fatal rhabdomyolysis and other unwanted effects that limit compliance. Development and testing of new agents with increased efficacy, improved safety profiles and increased compliance is still of major importance to clinicians, drug companies and governments. Elk velvet antler (EVA) has been reported to possess efficacy in improving lipid profiles as well as in attenuating blood coagulation. Establishing the effectiveness and safety of EVA could lead to a major advancement in the treatment of coronary artery disease.

Elk antlers or antlers from other cervids, such as deer and moose, are appendages of the skull created from a thick bony core which is upheld on permanent skin covered pedicles (Church, 1998, Goss, 1983). Antlers, unlike horns, are cast off every year and

then must be regrown through a process of regeneration, proliferation and mineralization (Sim, 1998). The developing antler is composed of an aggregate of immature cells that will differentiate into cartilage, calcified cartilage and finally bone (Banks and Newbrey, 1983a, Bank and Newbrey, 1983b). Velvet refers to the skin covering these differentiating cells as it describes the velvet texture provided by the dense growth of fine hairs on the skin surface (Sim, 1998, Woodbury, 2000). The terms, "velvet" and "velvet antler" are used interchangeably in reference to developing antler (Woodbury, 2000). At this early stage of development where the antler is soft and lacks mineralization, the entire antler is harvested, dried and processed for therapeutic applications.

The unique health beneftis associated with the velvet antler are caused by the biochemicals that regulate cellular differentiation and growth and that allow antler to be the most rapidly growing organ in adult mammals (Sim, 1998). During the rapid growth phase antler contains, collagen, lipids, glycosaminoglycans, omega-6 fatty acids, amino acids, calcium, phosphate, iron, zinc, prostaglandins, chondroitin sulfate, alkaline phosphatase, and growth factors (Church, 1998). Traditional medical reports and clinical observations from asia suggest that these components of antler influence body metabolism in general (stimulating and tonic), protect and restore damaged organ tissues (accelerate healing and recovery from injury), promote immune and phagocytic functions, slow the aging process, improve cardiovascular function, lower plasma cholesterol, inhibit blood coagulation, inhibit liver toxicity and enhance gonadotropic and thyroid function (Sim, 1998; Wang, 1988). Specifically, studies have reported the beneficial effect of velvet antler on plasma cholesterol and lipid profiles (Ahn, 1994; Breckham, 1969), as well as an anticoagulant effect (Church, 1998).

The objective of this study was to examine the dose dependent effects of oral administration of EVA on plasma lipid profiles, development of athereosclerosis and blood clotting time in diet-induced hypercholesterolemic rabbits.

This animal model of hypercholesterolemia has been established as an effective model for atherosclerosis research (Jokinen et al., 1985; Fekete, 1992) and as well as for testing of antihyperlipidemic and anticoagulant agents (Bocan, 1998; Koboyashi et al, 1989; Ishida et al., 1990; Kawashima et al., 1997; Mitropoulos et al., 1987). Three oral doses of EVA were tested against, a regular diet control, a 1% cholesterol diet control, a sham control using three doses of bone meal, an established antihyperlipidemic agent, fluvastatin and an established anticoagulant, warfarin.

Materials and Methods:

All animal experiments performed within this study were approved by the Animal Ethics Committee at the University of Saskatchewan in accordance with the Guide for Care and Use of Laboratory Animals prepared by the National Institute of Health.

70 adult male New Zealand white rabbits weighing 2.5 - 3 kg were acclimatized for 2 weeks at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan. Rabbits were fed a regular rabbit feed obtained from Federated Co-op, (Saskatoon, SK). During the 4 week induction phase animals in groups 2 to 10 were fed regular rabbit feed with 1% cholesterol added, rabbits in group 1 received regular rabbit feed without added cholesterol. Each group was comprised of 7 rabbits. Purified

cholesterol was obtained from Sigma (Mississauga, ON). The feed pellets were prepared at the Alberta Agriculture, Food and Rural Development, Food Processing Centre (Leduc, AB). Rabbit were maintained on this diet for 4 weeks with food consumption and weights being monitored daily and weekly respectively.

At the end of the 4 week period blood samples were taken and analyzed. Complete blood cell counts and lipid profiles were done the same day at the Department of Laboratory Medicine, Royal University Hospital (Saskatoon, SK). Blood coagulation assays were performed in the Department of Pharmacology using a Coagulation Analyzer and assay kits provided by Dade Behring (Newark, DE).

Following the 4 week induction period animals were given diets containing test agents. The following list indicates which test agent each group received with doses being listed as mg dose per kg body weight per day.

Group 1	Control with regular rabbit feed
Group 2	1% cholesterol diet control
Group 3	10 mg/kg/day EVA in 1% cholesterol diet control
Group 4	30 mg/kg/day EVA in 1% cholesterol diet control
Group 5	100 mg/kg/day EVA in 1% cholesterol diet control
Group 6	10 mg/kg/day bone meal in 1% cholesterol diet control
Group 7	30 mg/kg/day bone meal in 1% cholesterol diet control
Group 8	100 mg/kg/day bone meal in 1% cholesterol diet control
Group 9	1.2 mg/kg/day fluvastatin
Group 10	0.1 mg/kg/day warfarin

These diets were also prepared at the Food Processing Centre (Leduc, AB). Rabbits were to be fed these test diets for a period of 12 weeks. At the end of the 12 week period blood samples would be obtained and aortas excised for assessment of lipid deposition. To assess atherosclerosis, aorta segments were immersed in 10% buffered formalin solution for 24 h and then rinsed briefly in 70% alcohol. Tissues were then sliced longitudinally and immersed in Herxheimer's solution that contained Sudan iV (5 gm), ethyl alcohol 70% (500 ml) and acetone (500 ml) at room temperature for 15 min and washed in running water for 1 h. Digital photographs were taken with stained areas being quantified using a densitometer computer program (Beckman).

Results:

There were no significant differences in rabbit body weight observed during the four month induction diet period. The results for Groups 2 to 10 have been pooled as each group received the same diet and no significant difference was observed for any measured parameter between these groups. Figure 1 shows the results from the complete blood count and lipid profile. White blood cell (WBC) counts were elevated in rabbits receiving 1% cholesterol but no other signs of infection were observed. Red blood cell (RBC), hemoglobin, hematocrit and platelets showed no significant change. Lipid profiles showed a significant increase in total cholesterol, HDL cholesterol and the ratio of total cholesterol to HDL cholesterol. Triglyceride levels were not significantly different.

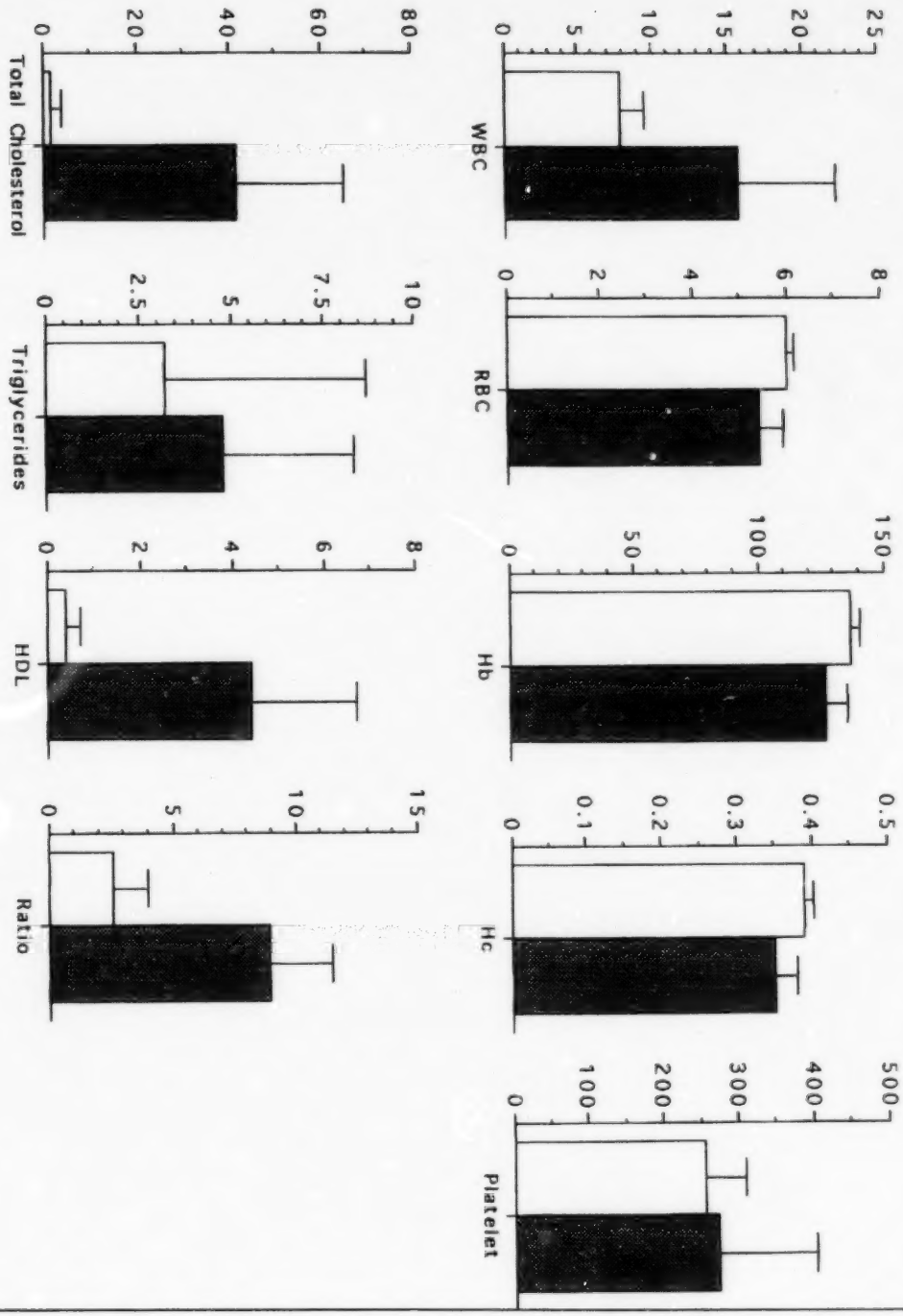

Figure 1: Graphs show the mean ± SD for Group 1 (open box) and Groups 2 to 10 (shaded box). WBC, White blood cells (10^9 cells/L); RBC, Red blood cells (10^{12} cells/L); Hb, Hemoglobin (g/L); Hc Hematocrit (L/L); Platelet (10^9 cells/L); Cholesterol and Triglycerides (mmol/L). WBC, Total Cholesterol, HDL and Ratio are all significantly elevated ($p<0.05$, *T-test*)

Coagulation assays showed no significant difference between Groups 1 and Groups 2 to 10. Table 1 shows values obtained from three coagulation assays Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), and Thrombin Time (TT).

Table 1

	Group 1 (Mean ± SD)	Group 2 - 10 (Mean ± SD)
APTT (sec)	16.7 ± 8.4	8.9 ± 8.9
PT (sec)	8.1 ± 3.9	6.4 ± 4.5
TT (sec)	12.1 ± 3.1	8.4 ± 4.2

The test agents were started immediately following the four week induction phase, with 1% diets containing test agents. Group 1 rabbits remained on regular feed and Group 2 received only 1% cholesterol feed without any test agents. One week after starting the test phase, animal care techinicians involved in the study noticed four rabbits had stopped eating. From their overall appearance the suspected cause was a gastric trichobezoars (hairballs) or constipation. As the animals were not eating and therefore not consuming the test agents, they were removed from the study. The rabbits were from Group 1, 9 and two from Group 10. One week later 18 more animals were observed to have stopped eating and on the next day showed signs of jaundice. Animals were sent for necropsy analysis. As more animals became ill the study was terminated at 9 weeks, 3 weeks before the scheduled termination date with only 31 rabbits remaining in the study. All but two of the test groups were down to 3 or fewer animals. The only groups to have 5 of 7 animals remaining were Group 9 (fluvastatin) and Group 5 (100 mg/kg/day EVA) (Figure 2).

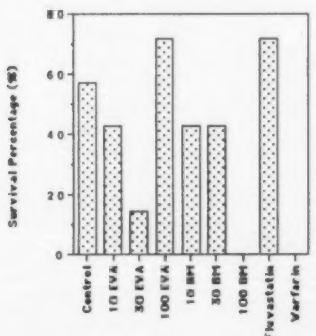

Figure 2: Percentage of animals which had not not stopped eating or shown signs jaundice after 9 weeks of test diet. Each group had seven animals at the start of test agent phase. No value was significantly different.

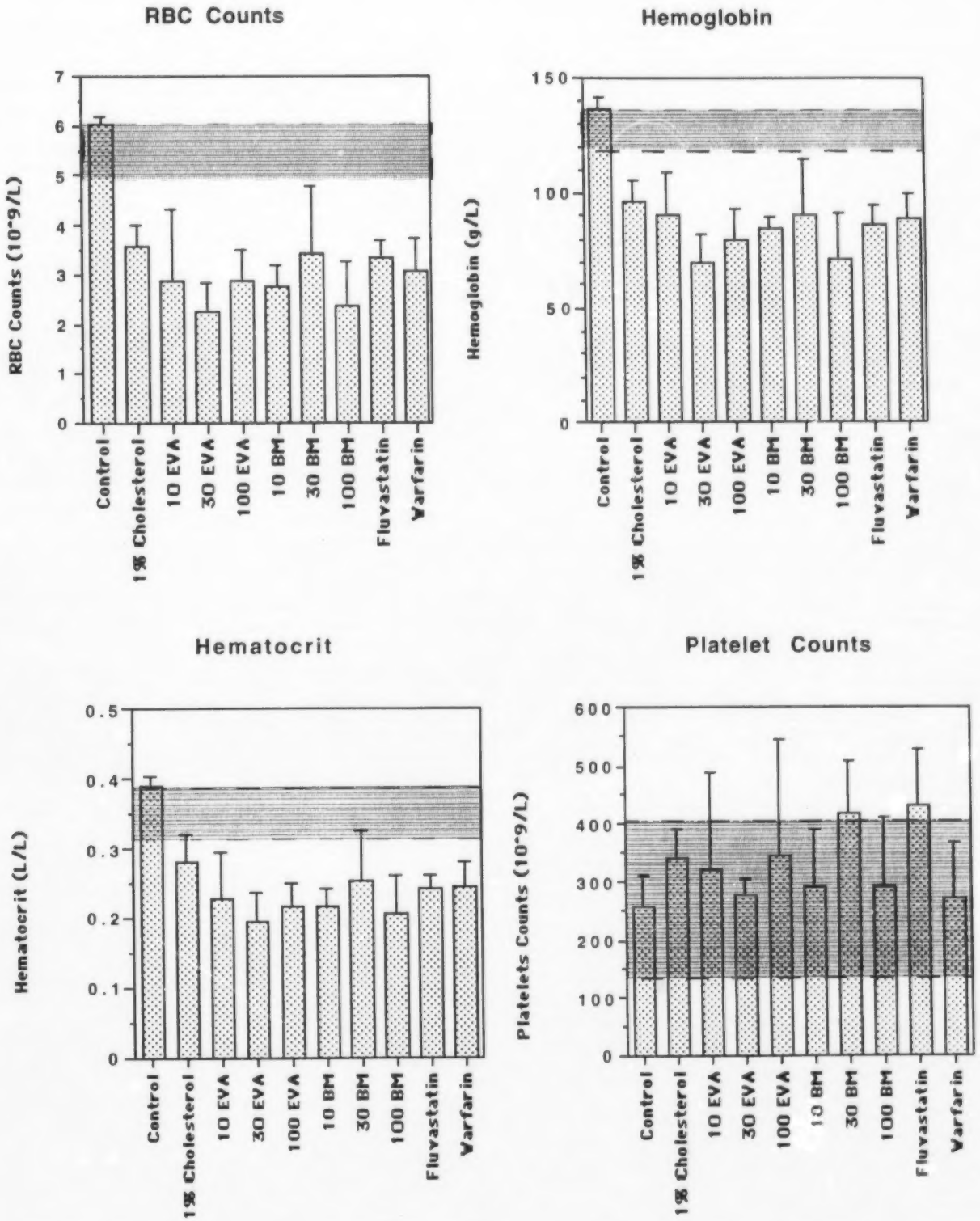

Figure 3: Blood count analysis for rabbits following 9 weeks of test diets. Shaded areas indicate range of values from 4 week induction phase.

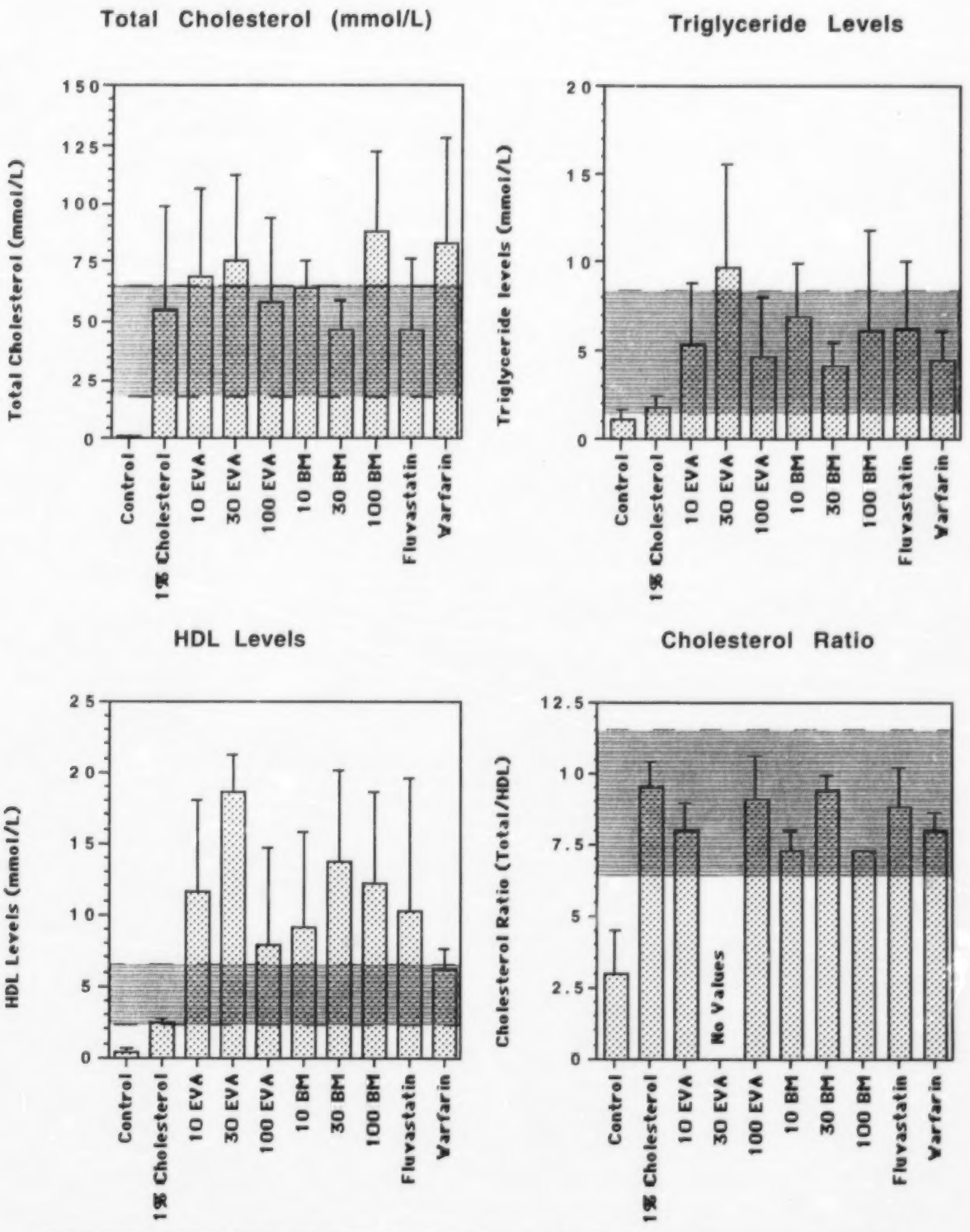

Figure 4: Lipid profile analysis for rabbits following 9 weeks of test diets. Shaded areas indicate range of values from 4 week induction phase.

Final blood sample analysis revealed an expected decrease in RBC counts, hemoglobin and hematocrit due to the hemolytic anemia (Figure 3). Lipid values remained significantly elevated with none of the treatment groups showing a therapeutic reduction in cholesterol values (Figure 4). The elevations observed for HDL cholesterol were not significant although it appeared several group had raised HDL levels.

As a result of the severe hemolytic anemia and small number of animals remaining in the study, coagulation assays were not preformed on the final blood samples. Aortas were removed and stained to assess deposition. Densitometer analysis of stained lipid deposits showed no significant difference between treatment groups.

Discussion:

Several studies have been conducted on New Zealand white rabbits using 1% cholesterol and the cholesterol levels observed in this study appear to correlate within the reported ranges (Mitropoulos et al., 1987; Bailey et al., 1994; Bocan et al., 1998; Jorge et al., 1994). The coagulation results appear to indicate decreased clotting time however these decreases were not significant. Although an increased clotting effect has been observed in hypercholesterolemic humans (Chan et al., 1996; Boon et al., 1995), the few studies that have been conducted in hypercholesterolemic rabbits support our results of no change in clotting times (Kawashima et al., 1997; Mitropoulos et al., 1987). These reports suggest although there are measurable changes in clotting factors which would suggest changes in clotting times other factors or mediators, such as protein C, seem to compensate and prevent changes in clotting times. *In vivo* coagulation due to platelet activation by collagen found on the atherosclerotic plaque surface should still be elevated (Boon et al, 1995). A future study directly examining thrombus formation *in vivo* would help distinguish the effects on clotting of hypercholesterolemia.

The results from the test phase of this study are difficult to interpret due to the hemolytic anemia and hepatic fibrosis that occurred in a majority of animals. Necropsy reports identified three main processes;

i) The atherosclerosis and lipidosis associated with the elevated cholesterol.
ii) Hepatic fibrosis, bile stasis and jaundice
iii) Regenerative hemolytic anemia, hyperbilirubinemia and hemoglobinuria

All three of these effects maybe due to the increased cholesterol causing hepatic lipidosis which would lead to bile stasis and fibrosis and also increased red blood cell membrane cholesterol leads to fragile and irregular shaped cells causing the anemia. There were no signs of infection or infectious agents, and there was no evidence of a auto-immune hemolysis. Initial toxin screens for zinc and copper, two metals known to cause hemolytic anemia, were negative. Other studies conducted using 1% cholesterol reported no adverse events (Mitropoulos et al., 1987; Bailey et al., 1994; Bocan et al., 1998; Jorge et al., 1994). Higher concentrations of cholesterol (5%) do result in hemolytic anemia but the extenisve liver fibrosis and jaundice were not reported (Akahane et al., 1986; Lichenstein et al., 1987). Toxicological evaluations of the feed will be done by the Toxicology Centre, University of Saskatchewan to determine if a any toxins or unidentified substances are present. We hope that with further data we will be able to publish a report on the cause of this anemia and liver toxicity. Although the suvival data

was not statistically significant the highest rates of survival were in groups receiving either 100 mg/kg/day EVA or fluvastatin. To determine if EVA may have a protective effect on liver function we have undertaken a study in Dr. S. Hemmings laboratory in the Department of Physiology, University of Saskatchewan. This study will examine the hepatic protective effect of oral EVA to known hepatotoxins.

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